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Topical Review

Ion Channels as Molecular Coulter Counters to Probe Metabolite Transport

S.M. Bezrukov

Laboratory of Physical and Structural Biology, NICHD, National Institutes of Health, Bethesda, Maryland 20892-0924, USA and
St. Petersburg Nuclear Physics Institute, Gatchina, Russia 188350

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Introduction

Traditionally, the functional properties of transmembrane channels have been approached in terms of small-ion conduction. Since the inception of two groundbreaking techniques to observe single channels, namely, channel reconstitution into planar bilayers (Bean et al., 1969; Miller, 1986) and patch-clamping (Neher & Sakmann, 1976; Sakmann & Neher, 1995), the same basic electrophysiological methods have been applied to two, distinctively different groups of channels. These are the 'small,' highly ion-selective channels of excitable membranes of neurophysiology and the 'large,' poorly ion-selective channels that include bacterial porins, mitochondrial channels, gap junctions, some toxins, the nuclear pore complex, and protein-conducting channels in the endoplasmic reticulum. High-resolution monitoring of single-channel currents has proven to be a major tool in studies of mechanisms by which neurophysiological channels gate and select between different ions (Hille, 1992).

Large channels seem to be designed by Nature to serve a different purpose. This is, most probably, to regulate the passive and active transport of molecular species that are larger than halide, alkaline, or alkaline earth metal ions. These large channels are pathways for metabolites and macromolecules such as proteins and nucleic acids. At the same time, they can pass small ions. By measuring the current carried by the small ions one can access not only the structural changes in the channel resulting from processes such as voltage or ligand gating, but also the transport of larger molecules through their transient occlusion of ion current. Otherwise, direct large-molecule flux determination requires heroic effort with multipore membranes (or even whole cells and organelles) and long observation times using radioactive, fluorescent, or colorimetric probes (Weisman et al., 1989), electron-opaque tracers (Feldherr, 1986), liposome swelling (Jap & Walian, 1990), or solute-specific reactions, such as the luciferin-luciferase system for ATP detection (Rostovtseva & Colombini, 1997).

Here I review the new approach to study channel-facilitated[1] metabolite transport. In this approach fluctuations in single-channel currents reveal large-solute partitioning into and dynamics within an ion channel aqueous pore. The change in resistance of an electrolyte-filled capillary produced by the passage of micron-sized particles has long been used for particle counting and sizing. A conventional device for such measurements, the Coulter counter, that makes use of an orifice of about 30 microns in diameter, is able to detect particles down to several tenths of a micron (Kubitschek, 1958; Allen, 1967; Bunville, 1984). Nuclepore technology (in which orifices are etched particle tracks) extended the lower size limit by detecting 60 nm particles in a 0.45-μm-diameter capillary (DeBlois & Bean, 1970; DeBlois et al., 1977).

Correspondence to: S.M. Bezrukov

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[1] A discussion of direct solute permeation through the lipid bilayer rather than through channels can be found elsewhere (Volkov, Paula & Deamer, 1997; Paula & Deamer, 1999).

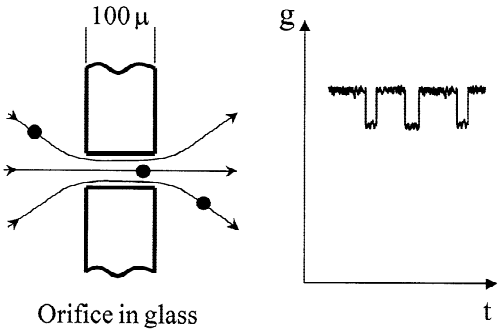

Fig. 1. In a conventional Coulter counter the flow of liquid maintained by an external pressure difference drives micron-sized particles through the sensor capillary in a glass partition. Each particle entry produces a pulse in the capillary conductance. The number of pulses per second reports on the particle concentration; the particle size can be inferred from the pulse amplitude.

Now 'molecular technology' increases the potential capacity of the method still further. The aqueous pore of a large channel in its completely open conformation can be used to detect passage of molecules with effective radii of several angstroms only. The 'molecular Coulter counter' (Bezrukov, Vodyanoy & Parsegian, 1994) allows quantitative analysis of the dynamics and partitioning of higher molecular weight molecules such as metabolites and macromolecules in the presence of small permeant ions. This is potentially a very general method applicable to a wide range of physiologically important solutes and a variety of different channels.

The Coulter Principle

The underlying idea of the resistive pulse technique that has been used in Coulter counters since the early fifties (Coulter, 1953) can be formulated as follows. If a nonconducting particle suspended in a conducting medium moves into a small capillary, it increases the resistance of the capillary relative to that of the capillary filled with the conducting medium alone (Fig. 1). The magnitude of this increase in resistance is related to the particle size. In conventional Coulter counters the particle suspension is made to flow by a hydrostatic pressure difference maintained across the capillary, so that the flow velocity defines the duration of the resistance increase.

The changes in the resistance as particles pass through the capillary generate voltage pulses, which are amplified, sized, and counted. The technique was first applied to blood cell counting, but with subsequent modifications (Kubitschek, 1958) it permitted counting of bacterial cells and could be applied to the measurement of cell-volume distributions. With further developments, the method has become very popular in a wide range of applications from medicine to geology (Allen, 1975; Kachel, 1979).

Based on original Maxwell's (1904) considerations, the decrease in the conductance of a long capillary of radius R and length L upon entry of a single particle of radius $r \ll R$ can be expressed as (Gregg & Steidley, 1965)

$$\Delta g_s = \frac{2\pi r^3 \sigma}{L^2} \tag{1}$$

where σ is solution conductivity. If the particle and channel radii are comparable, the effect is larger (DeBlois & Bean, 1970; DeBlois et al., 1977). The concentration of particles can be adjusted in a way that the probability of two particles being in the capillary at the same time will be low, so that each particle passage will produce a conductance change described by Eq. 1. It will result in a sudden current (voltage) pulse of corresponding amplitude. Equation 1 demonstrates that the amplitude of the pulse is proportional to the third power of particle radius, that is, to particle volume. Taking into account noise considerations, this sets the limit on particle size detection at about 2% of the capillary diameter. Particles with the size below this value tend to be swamped by electric noise. For a conventional counter with the smallest aperture of about 30 μm this means the lower limit of about 0.5 μm.

The duration of a pulse can be calculated from the system hydrodynamics. In the case of a not very short capillary it can be estimated by the characteristic time that takes the fluid to pass the capillary along its axis under the laminar flow conditions

$$\tau_{flow} = \frac{4\eta L^2}{\Delta P R^2} \tag{2}$$

where η is the fluid viscosity and ΔP is the hydrostatic pressure difference. At optimal conditions a Coulter counter can count and classify these pulses according to their size at a rate up to 5,000 per second.

An extension of the resistive pulse technique capabilities to size particles much smaller than 1 μm was achieved with the help of the sensor consisting of a single pore in a Nuclepore membrane (DeBlois & Bean, 1970; DeBlois et al., 1977). The sensor is prepared by passing cast polycarbonate film across a heavy nuclear particle source. Tracks left in the film are then etched chemically to provide a precise pore size in a micron-submicron range. With pore dimensions of tenths of a micron in diameter and 1–2 microns in length, the Nuclepore technology has been successfully applied to count viruses and bacteriophage particles down to 60 nm.

Using conductance noise analysis (Bezrukov, Drab-

kin & Sibilev, 1986) rather than single pulse detection, even higher sensitivity can be achieved (Bezrukov, Pustovoit & Sibilev, 1992). The source of useful signal in this case is random fluctuations in particle number density. The sample volume is effectively 'scanned' by the capillary, so that fluctuations in the particle number generate noise in the capillary conductance, *g*. With the help of a macroscopic capillary of about 30 μm in diameter and half a millimeter in length it was possible to size sodium dodecyl sulfate (SDS) and eycosanethylene glycol octadecyl monoether ($C_{18}E_{20}$) micelles of about 5–10 nm in size. Interestingly, the factor limiting sensitivity in this method is noise generated by ion number fluctuations (Feher & Weissman, 1973; van den Berg et al., 1986; Bezrukov et al., 1989). The normalized signal, $\langle(\delta g)^2\rangle/g^2$, from spherical particles of radius r occupying volume fraction F does not depend on electrolyte concentration and equals $3\pi r^3 F/V$, where V is the capillary volume (Bezrukov et al., 1992). At the same time normalized noise stemming from ion concentration fluctuations is given by their inverse total number in the sample (though not for electrolyte mixtures (*see* Bezrukov et al., 1989), and equals $1/2nV$, where n stands for electrolyte concentration (molecules per volume). Equating these two values, we obtain

$$r_{min} = \sqrt[3]{\frac{1}{6\pi}\frac{1}{nF}}. \tag{3}$$

For 1 M 1–1 electrolytes and particle volume fraction of 1% this relation gives $r_{min} = 2$ nm. Conductance fluctuations from smaller particles at this volume fraction and electrolyte concentration are hard to resolve because of ion number fluctuations.

Going to Molecular Scale: Three Size-related Issues

Large, mesoscopic ion channels such as mitochondrial channels, bacterial porins, several toxins, gap junctions, the nuclear pore complex, and protein-conducting channels in the endoplasmic reticulum are good candidates to serve as molecular sensors or 'molecular capillaries' in the resistive pulse technique (Fig. 2). Their water-filled pore diameters are in the range of one to several nanometers. What are main consequences of going from micron to nanometer scales? What are the basic differences between the classic version of a Coulter counter briefly discussed above and its molecular version using ion channels? Here we will discuss the following three size-related issues:

(i) Predominance of diffusion at small scales.
(ii) Growing contribution of particle charge.
(iii) Increasing role of particle interactions with capillary walls.

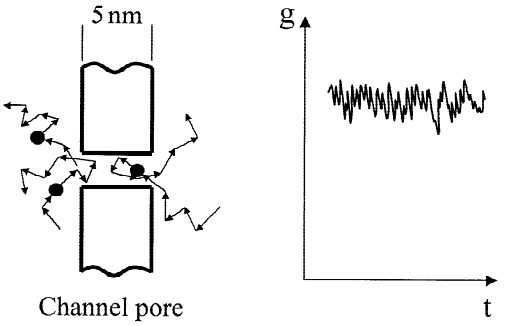

Fig. 2. In the molecular version that uses an ion channel pore as a sensor, the directional hydrodynamic flow is negligible for all reasonable hydrostatic pressure differences. Diffusion dominates particle exchange. In the absence of strong particle-pore interactions individual pulses are too short to resolve. To recover particle dynamics, spectral analysis of the pore conductance noise must be used.

Directional Flow Vs. Diffusion

It is very well known that 'diffusion velocity,' that is the distance traveled by a diffusing particle divided by the observation time, is a function of the observation time (e.g., Berg, 1993). The smaller is this time, the higher is the diffusion velocity. As a consequence, the characteristic time of diffusion decreases faster than the distance. In the case of one-dimensional diffusion the mean square distance $\langle x^2\rangle$ and the observation time t_0 are related through diffusion coefficient D by

$$t_0 = \frac{\langle x^2\rangle}{2D}. \tag{4}$$

Though this formula does not describe exactly the relaxation time for the diffusion-driven exchange of particles between the bulk and the capillary (*see* next section), it can be used to crudely estimate out-of-capillary diffusion time if we substitute $\langle x^2\rangle$ with L^2. For typical sizes of particles that are accessible for studies using a conventional Coulter counter (about a micron) and capillary lengths (tens of microns), this time is too large to interfere with the device operation. For example, for a capillary of 10 μm in length containing 1 μm particles in water under normal conditions, the diffusion time given by Eq. 4 is about one hour. It should be compared with one millisecond characteristic pulse time, which is defined by the directional fluid flow through the capillary of a conventional Coulter counter. In fact, it is very hard to observe a purely diffusional regime of particle exchange in the case of macroscopic capillaries, as even a very small, uncontrollable, of about 1 μm water column hydrostatic pressure differences will introduce flow-induced motion with transient times that are smaller than that given by Eq. 4.

When the dimensions of the system go down to mo-

lecular sizes, the situation changes qualitatively and, actually, becomes reversed. It is seen from comparison of Eq. 2 with Eq. 4. As the capillary sizes, L and R, and the particle size r, (the particle must fit the capillary) get smaller, the laminar flow time given by Eq. 2 stays constant while the diffusion time decreases as the third power of the size. An additional factor amplifying the relative rate of the Brownian motion is an increase in a particle diffusion coefficient as the particle size decreases. Indeed, if all sizes are scaled down in the same proportion particle diffusion coefficient in Eq. 4 changes as $D \propto 1/r \propto 1/L$, so that $t_0 \propto L^3$. Using a more exact expression for the diffusional relaxation time, $\tau_{diff} = L^2/12D$ (*see* next section), we can estimate the ratio of the characteristic times as

$$\frac{\tau_{diff}}{\tau_{flow}} = \frac{\Delta P R^2}{48 D \eta}. \tag{5}$$

It is easy to show now that for a molecular pore the Brownian motion of particles dominates over directional flow at all reasonable hydrostatic pressure differences across the system. To do that we put $\tau_{diff} = \tau_{flow}$ and solve Eq. 5 in respect to ΔP. That is, we ask: what is the hydrostatic pressure difference that would allow the flow-driven particle exchange rate to be comparable to the diffusion-driven one? Substituting the following values: $R = 1$ nm, $\eta = 10^{-3}$ kg/msec (viscosity of water at 20°C), and $D = 3 \times 10^{-10}$ m^2/sec (e.g., ATP diffusion coefficient in water (Diehl, Ihlefeld & Schwegler, 1991)), we obtain $\Delta P \cong 1.5 \times 10^7$ Pa, which is equivalent to a one-and-half-kilometer-high water column. It means that only at pressure differences higher than this one the exchange of molecules will be governed by flow. At smaller pressure differences diffusion dominates.

Our estimations used several simplified assumptions. The most important assumption is that we have taken the particle diffusion coefficient in bulk water samples to describe diffusion in an ion channel pore. We will return to this question later. However, even with this simplification, our estimate is reliable enough to demonstrate the qualitative change in the system behavior and to highlight the importance of diffusion on the molecular scale.

Nonconducting Particle Vs. Polyion

The formula relating the magnitude of conductance reduction to the electrolyte conductivity and particle size, Eq. 1, was repeatedly verified experimentally for micron-sized objects (e.g., Allen, 1967; DeBlois & Bean, 1970; DeBlois et al., 1977). Does it hold true for the objects of molecular size when they are smaller than the mean distance between electrolyte ions (about 10 nm for 1 mM 1–1 electrolytes)? To check this, we need a test object of molecular size that does not bear any charge and does not bind water molecules too tightly. In the case of charged molecules we can expect complications related to the additional conductance introduced by the test object itself; in the case of strong water binding the 'electrical' dimensions of the test object can differ from purely geometrical ones.

It seems like the best test object is the water molecule itself. The necessary 'experiment' can be done very quickly. We first note that Eq. 1 permits us to calculate particle size using conductivity data on a condition that particle concentration is large enough to introduce measurable effects, but small enough to neglect particle-particle interactions. Indeed, consider a conductometer cell of unitary dimensions. Its conductance in the absence of particles will be numerically equal to electrolyte conductivity σ. Adding particles at concentration n_p will decrease this conductivity by $\Delta\sigma = n_p \Delta g$ where Δg is given by Eq. 1 with $L = 1$. Dividing this relation by σ and solving it with respect to r we have

$$r = \sqrt[3]{\frac{1}{2\pi n_p}\frac{\Delta\sigma}{\sigma}}. \tag{6}$$

Now, adding 1 ml of water to 1 liter of a salt solution, we will change its conductivity by 0.1%. The concentration of added water molecules, the test objects, will be 3.3×10^{25} m^{-3}. Substituting these numbers in Eq. 6 we have $r_w \cong 0.17$ nm, which gives a very good estimate for the water molecule radius.

It turns out that even charged molecules can be sized this way if the small-ion concentration is high enough (Rostovtseva & Bezrukov, 1998). For example, ATP molecules at neutral pH carry four elementary charges each so that their addition to a diluted sodium chloride solution increases its conductivity. However, at high sodium chloride concentrations electrolytic features of ATP are progressively suppressed and its interference with current curried by small ions (Na^+ and Cl^-) is made evident. Figure 3 shows that at low sodium chloride concentrations ATP behaves as an electrolyte, whereas at concentrations higher than 0.5 M NaCl, ATP molecules act as nonconducting particles that decrease solution conductivity. The effect is close to saturation at 2 M NaCl. Formally applying Eq. 6 to this point we obtain $r_{ATP} \cong 0.77$ nm, which is in accord with the hydrodynamic radius found from ATP diffusion coefficient in water (Diehl et al., 1991). Thus, in an analogy with optics, dual properties of ATP-sized charged molecules can be contrasted by diluting or concentrating small-ion solutions.

Particle-Pore Interactions

Going to molecular scale we encounter different kinds of interactions between the particle and the pore which

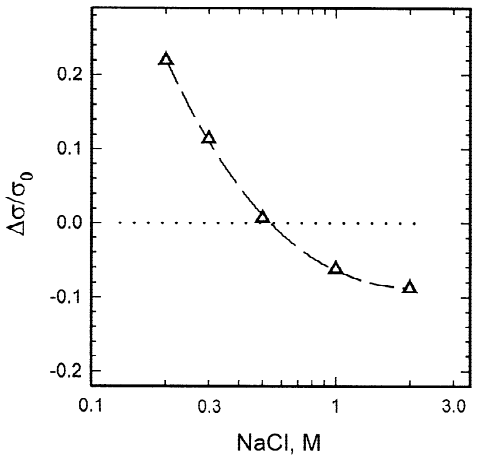

Fig. 3. Electrolyte or nonconducting particle? Dual properties of ATP are seen in the effect of 50 mM ATP addition on small-ion conductivity. At NaCl concentrations smaller than 0.5 M, ATP increases bulk solution conductivity — the electrolytic feature of ATP is evident. At higher NaCl concentrations, addition of ATP reduces specific bulk conductivity — ATP molecules act as nonconducting particles interfering with small-ion flow. From Rostovtseva and Bezrukov (1998).

hardly mattered in the case of a conventional Coulter counter. These interactions can be divided into steric, hydrodynamic, and electric. An intuitively clear picture of complete exclusion of a large hard sphere from a small pore gives the simplest example of steric interactions. However, even when the sphere radius is reliably smaller than the pore radius, these interactions can still be significant. A straightforward geometrical consideration (e.g., Colton, Satterfield & Lai, 1975) shows that in the case when the sphere radius is half the pore radius, the partitioin coefficient is reduced by a factor of four. For flexible polymers, steric interactions are mostly related to the loss in the number of possible polymer configurations as a result of its confinement by the pore (de Gennes, 1979; Grosberg & Khokhlov, 1994). This entropic repulsion plays an important role in polymer partitioning into the channel pore and, therefore, in polymer transmembrane transport.

Hydrodynamic interactions include several effects, first of all, the propulsion of water inside the channel by a diffusing particle and additional sheer friction arising between channel walls and the particle surface. If the particle and pore sizes are comparable, this interaction results in a substantial decrease in diffusion rate (Bean, 1972). In addition, in the case of molecular sizes, the state of water at the interface could be different from its bulk state (Rabinovich & Derjaguin, 1988; Berkowitz & Raghavan, 1994) and the channel inner surface may be of no exception. This brings us to the question about the value of 'water viscosity' that should be used to calculate the diffusion rate in a 1 nm channel.

The importance of electric interactions is also dramatically increased by the micron-to-nanometer scale transition. These interactions are not restricted to just Coulomb forces between fixed charges on the pore wall and on the metabolite molecule. They necessarily include van der Waals attractive forces and multiple electrostatic polarization effects. A wealth of research in surface and colloid science (Parsegian et al., 1986; Ninham, 1989; Zydney & Pujar, 1998) is immediately available to think about various mechanisms of solute selectivity based on electrical interactions.

Particle Behavior from Conductance Fluctuations

Among the three size-related features discussed above, the most instructive is the predominance of diffusion at small scales. It allows approaching the question of 'pulse duration' by solving a purely diffusional problem.

Conductance noise arising from the stochastic nature of diffusion of matter or heat has been repeatedly addressed for at least the last 50 years (Richardson, 1950; van Vliet & Fasset, 1965; Weissman, 1988). To relate a characteristic 'cutoff' frequency (frequency bandwidth) of diffusion noise in the conductance of a capillary channel of length L ($L \gg R$) in the presence of particles with the diffusion coefficient D, different authors use different expressions: D/L^2 (Kim et al., 1980), $D/\pi L^2$ (van den Berg et al., 1986), or $6D/\pi L^2$ (which follows from the characteristic diffusion relaxation time, $L^2/12D$, given by Feher and Weissman (1973), if one assumes a simple Lorentzian shape for the noise spectrum). Calculation of the cutoff frequency and the characteristic diffusion relaxation time is closely related to the 'first passage time problem' widely discussed in chemical physics (e.g., Weiss, 1966). It is possible to proceed this way and to calculate the characteristic diffusion relaxation time using proper averaging over the capillary length. However, it is very well known that the spectra of diffusion noise generally have rather complex shape (Richardson, 1960; van Vliet & Fasset, 1965) and only under special circumstances are described by a single Lorentzian (Bezrukov & Vodyanoy, 1994). This means that the relaxation is nonexponential and, as a consequence, the relaxation time for diffusion is not as clearly defined as for a two-state Markovian process.

All estimates discussed above predict a very high noise bandwidth and, correspondingly, very short diffusion relaxation times. With the diffusion coefficient anywhere close to its bulk value for a 1–2 nm particle in water, we obtain the diffusion relaxation time in the range of nanoseconds. This time is too short to be resolved in single-channel experiments. Correspondingly, the cutoff frequency of power spectral density is too high (MHz range) to be measured. Nevertheless, the diffusion relaxation time and, therefore, the diffusion coefficient of the particle in the pore, can be deduced from the

low-frequency part of the fluctuation spectrum if certain additional information is available.

The idea is that the mean square conductance fluctuation is simply a product of the square of the pore conductance change produced by entering of a single particle, Δg_s, and the average number of particles in the pore, $\langle N \rangle$, that is, $\Delta g_s^2 \langle N \rangle$. On the other hand, it is a product of the noise low-frequency spectral density, $S(0)$, and the effective noise bandwidth, f_n, obtained from the diffusion model and expressed through the diffusion coefficient and pore length. Thus, measuring $S(0)$ one can deduce the diffusion coefficient of the particle in the pore. Leaving out the details, we obtain (Bezrukov et al., 1994; Rostovtseva & Bezrukov, 1998)

$$D = \Delta g_s \langle \Delta g \rangle L^2 V^2 / 3 S_i(0), \tag{7}$$

where $\langle \Delta g \rangle$ stands for the average particle-induced reduction in pore conductance seen as the reduction in the mean single-channel current, V is the transmembrane voltage, and $S_i(0)$ is the low-frequency power spectral density of particle-induced current fluctuations. Main assumptions include a long ($L \gg R$) homogeneous pore and the absence of any particle–particle interactions. The second assumption is always fulfilled in the case of small $\langle N \rangle$.

While Eq. 7 describes only the low-frequency part, the complete spectrum can be expressed through an infinite sum of properly weighed Lorentzians. This sum gives 'white' (frequency-independent) behavior at low frequencies and the characteristic '$f^{-3/2}$ diffusion slope' (Richardson, 1950; van Vliet & Fasset, 1965) at high frequencies. The value of the diffusion relaxation time depends on the definition of the characteristic time for nonexponential relaxation. Within its assumptions Eq. 7 is exact, but the corresponding relaxation time can be equal to $L^2/6\pi D$, $L^2/12D$ (the result given earlier by Feher and Weissman (1973) and Berg (1993)), or $L^2/\pi^2 D$, depending on the choice of the definition.

Flexible Neutral Polymers in Channel Pores

Flexible water-soluble polymers are promising tools in studying ion channel properties in their functional states (Zimmerberg & Parsegian, 1986; Krasilnikov et al., 1992; Bezrukov & Vodyanoy, 1993; Vodyanoy et al., 1993; Bezrukov et al., 1994; Parsegian et al., 1995; Bezrukov et al., 1996; Bezrukov & Kasianowicz, 1997; Kaulin et al., 1998; Merzlyak et al., 1999). Especially helpful polymer is poly-(ethylene glycol), PEG, because it can be dissolved in water in practically any proportion, is commercially available in many different sizes, and has a Kuhn length of several angstroms only. The latter property, associated with a relatively small size of PEG coils in water (Couper & Stepto, 1969; Kuga, 1981) is crucially important for data interpretation in polymer partitioning experiments. For the channels with pore radii in the nanometer range it allows analysis based on the scaling ideas of polymer science (de Gennes, 1979; Grosberg & Khokhlov, 1994), though generally the situation is more complex (e.g., Merzlyak et al., 1999).

Polymer partitioning into the channel aqueous pore is deduced from its effect on (average) channel conductance (Krasilnikov et al., 1992; Bezrukov & Vodyanoy, 1993). Though the mechanisms by which addition of neutral polymers reduces ion mobility are understood only qualitatively (Yam, Anderson & Buxbaum, 1988; Berezhkovskii et al., 1999), the usual assumption is that the effect of polymers on bulk electrolyte conductivity and pore conductance is the same and can be related to the average polymeric concentration. Therefore, the partition coefficient can be estimated as the ratio of the relative polymer-induced reduction of channel conductance to the relative reduction of bulk solution conductivity. Using this approach, it was found that while certain channels are able to accumulate molecules of small PEGs in their pores to an excess of PEG bulk concentration (e.g., Bezrukov et al., 1996; Bezrukov & Kasianowicz, 1997), others do not show any significant PEG accumulation (e.g., Bezrukov & Vodyanoy, 1993; Kaulin et al., 1998).

The molecular Coulter counter principle was first applied to alamethicin channels (Bezrukov & Vodyanoy, 1991; Bezrukov et al., 1994) whose PEG size-dependent filling does not show any attraction between the pore and the polymer. The absence of attraction is made evident by two observations. First, even the smallest PEGs change the amplitudes of channel currents to a smaller degree than the conductivity of the bulk phase (Bezrukov & Vodyanoy, 1993). Second, PEG decreases the relative probability of higher conductance states by collapsing the channel toward its closed state in agreement with the polymer-induced osmotic stress even for polymers of small, penetrating size (Vodyanoy et al., 1993).

In planar lipid bilayers alamethicin forms voltage-dependent channels that switch randomly between different conductance states (Hall et al., 1984). According to the most popular barrel-stave model, the average diameter of the channel changes from about 1 nm to about 2 nm for the first three conducting states (e.g., Sansom, 1991; Woolley & Wallace, 1992; Cafiso, 1994). From analysis of PEG-induced noise and PEG-induced conductance reduction (Bezrukov et al., 1994) it was inferred that the mobility of polymers inside the channel pore are reduced by a factor of about 20 in comparison with their mobility in the bulk. Small PEGs with molecular weights of 200, 300, and 400 Dalton showed higher mobility in higher conductance levels, but the decrease of the diffusion coefficient with the polymer molecular weight was much less expressed than in the bulk.

The reduction in mobility of a particle inside a pore

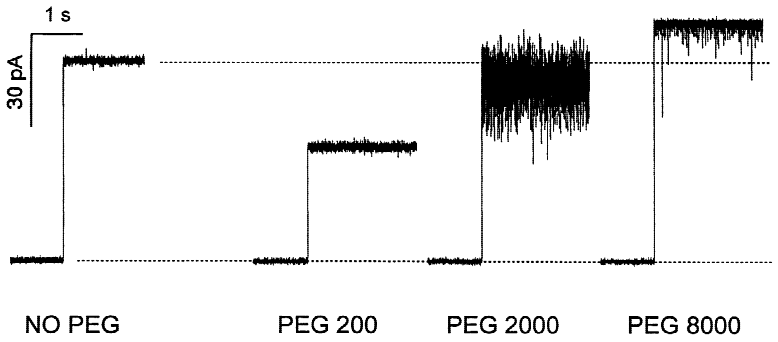

Fig. 4. Currents through a single α-toxin channel as affected by addition of PEG with molecular weights of 200, 2000, and 8000 Da. The control current trace on the left shows channel spontaneous insertion in the absence of polymer. The three traces on the right illustrate channel insertion in polymer-containing solution (1 M NaCl solution at pH 7.5 with polymers added to 15% weight/weight concentration for all PEGs). It is seen that while the blocking effect decreases as polymer molecular weight is increased (large polymers are sterically excluded from the pore), PEG-induced noise is maximal for PEG 2000. The applied potential was 100 mV, the signal was filtered by a 1 kHz low-pass Bessel filter. From Bezrukov et al. (1996).

of a comparable size can be explained by the restricted diffusion considerations. It is very well known that the diffusion rate measured as a total flux of molecular species through a synthetic membrane of a given pore area and thickness may be greatly slowed down if the dimensions of the pores are comparable with those of the diffusing molecules (for a review of early results *see* Papenheimer, 1953). The corresponding diffusion coefficient is less than the free diffusion coefficient and is termed the 'restricted diffusion coefficient,' D_{rd}. An analytical consideration of the problem was given more than half a century ago by Faxen (1922), who introduced the following correction to Stokes' law

$$\frac{D_{rd}}{D} = 1 - 2.10\frac{r}{R} + 2.09\left(\frac{r}{R}\right)^3 - 0.95\left(\frac{r}{R}\right)^5, \qquad (8)$$

where r and R are the particle and the capillary radii, correspondingly.

While the reduction in the diffusion coefficients for a polymer inside a molecular pore found in experiments with alamethicin (Bezrukov et al., 1994) is in a qualitative accord with the restricted diffusion theory, the absence of any significant dependence of the diffusion coefficient on the polymer molecular weight is not. In fact, it strongly disagrees with the restricted diffusion considerations for hard spheres that would predict a dramatic decrease in the diffusion rates as the radius of a diffusing sphere approaches the radius of a channel (Bean, 1972). This discrepancy probably reflects some differences in restricted diffusion of random flexible polymer coils and hard spheres. Polymer coils confined by a pore are limited in their possible space configurations. This confinement significantly distorts polymer initial (bulk) shape and is paid for by a decreasing partitioning of polymers as their size increases.

The partitioning and dynamics of PEGs in the confines of an ion channel pore were studied in numerous experiments with the *Staphylococcus aureus* α-hemolysin (α-toxin) channel (e.g., Krasilnikov et al., 1992; Bezrukov et al., 1996; Bezrukov & Kasianowicz, 1997; Merzlyak et al., 1999). The ability of this toxin to form channels in lipid bilayers, the main mechanism by which it is believed to damage target cells, was discovered some 20 years ago (Krasilnikov et al., 1981). Recently, the crystal structure has been determined to 0.19-nm resolution (Song et al., 1996). Contrary to the alamethicin channel studies, significant attraction between PEG and the α-toxin pore was found, especially at high salt concentrations. Two observations lead to this conclusion. First, there is a pronounced decrease of the channel conductance in the presence of small polymers. It exceeds PEG effect on bulk solution conductivity and suggests PEG accumulation in the pore to an excess of its bulk concentration. Second, there is a strong reduction of PEG mobility in the pore, which is seen as a high level of polymer-induced noise in the open channel current.

Figure 4 shows the effect of PEGs of different molecular weights on the current through a single α-toxin channel. The high noise level that is clearly displayed by the record for PEG with molecular weight 2000 Da reflects the slow rate of polymer translocation through the pore. The corresponding diffusion coefficient calculated from the power spectal density using Eq. (7) is several orders of magnitude smaller than its value in the bulk (Bezrukov et al., 1996). Given that PEG associates with hydrophobic regions of proteins (Arakawa & Timasheff, 1986), it shows that the lining of the aqueous cavity of the pore is, at least partially, hydrophobic. An additional factor accounting for the observed slow polymer translocation could be 'steric trapping' due to a bubble-like cavity in the α-toxin channel pore that was revealed in a crystallographic study (Song et al., 1996).

ATP Transport Through Mitochondrial Porin

The mitochondrial porin, VDAC (voltage-dependent anion channel) is known to provide a pathway for most of the metabolite flux across the outer membrane of mitochondria. The structural features (Mannella, Forte & Colombini, 1992; Song et al., 1998) and the ability to gate

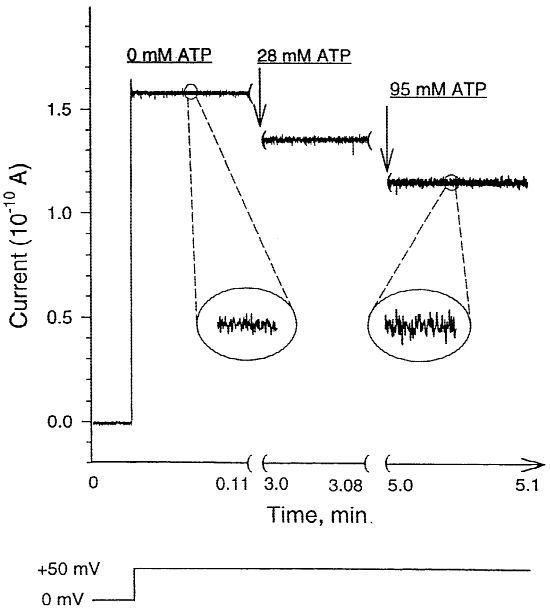

Fig. 5. Currents through a completely open VDAC channel in the presence of increasing amount of ATP (from left to right). Nucleotide addition to 1 M NaCl membrane-bathing solution reduces channel current and increases current noise (*see* insets that show currents at a finer scale) that is used to assess particle dynamics. VDAC was reconstituted into a planar lipid bilayer of 35 pF capacitance at room temperature and pH 8.0. Time resolution was 0.1 msec. The bottom trace indicates the potential applied to the membrane. From Rostovtseva and Bezrukov (1998).

between differently selective states (Hodge & Colombini, 1997) make this channel a good candidate for metabolite flux regulation. Results obtained on intact mitochondria suggested that VDAC could be responsible for nucleotide transport (Benz et al., 1988; Liu & Colombini, 1992; Gellerich et al., 1993; Benz, 1994; Lee, Zizi & Colombini, 1994). Recently, in experiments with VDAC reconstituted into planar lipid bilayer membranes (Rostovtseva & Colombini, 1997) it was directly demonstrated that VDAC is able not only to mediate but also to control ATP flux through the mitochondrial membrane. In the fully open conformation VDAC permeability for ATP is 1.1×10^{-20} m^3/sec, while in the closed state the permeability drops by two orders of magnitude. These measurements were performed on multichannel membranes using an ATP-specific reaction — the luciferin/luciferase method allowing detection of subnanomolar ATP concentrations.

The molecular Coulter counter approach was applied to a single VDAC channel whose conductance properties were studied in the presence of different concentrations of ATP (Rostovtseva & Bezrukov, 1998). Figure 5 demonstrates ATP-induced changes in the current through a single VDAC channel reconstituted into a planar lipid bilayer. Two effects of ATP application are immediately seen: a decrease in channel conductance and an increase in channel noise. As ATP concentration increases, the effect on channel conductance saturates at about 0.6 of the conductance initial magnitude, while the ATP-induced noise goes through a maximum at about 100 mM ATP in the bulk (*data not shown*). Channel conductance saturation to a nonzero value suggests that the channel conductance block by one ATP molecule is not complete. It turns out (Rostovtseva & Bezrukov, 1998) that it is actually close to the value predicted by Eq. 1. Analysis of the noise and conductance shows that mobility of the ATP inside the VDAC pore is reduced approximately by an order of magnitude in comparison to its bulk mobility. The diffusion coefficient of ATP in the channel is deduced to be $(1.6–3.3) \times 10^{-11}$ m^2/sec; the diffusion coefficient reported for bulk water solutions is 3×10^{-10} m^2/sec (Diehl et al., 1991).

The tenfold decrease in ATP mobility can be explained by the restricted diffusion theory. The correction described by Eq. 8 was widely used in molecular-sieve chromatography theory and had been experimentally shown to hold for r to R ratios as high as 0.3–0.4 (Renkin, 1955; Ackers, 1964; Casassa, 1971). For r/R ratio of 0.4 Eq. 8 predicts about fourfold reduction in diffusion coefficient. Later, a more exact calculation with accuracy up to the 10th power of r/R was performed and experimentally verified (for a review *see* Bean, 1972). It was shown that r/R ratio of 0.6 results in approximately tenfold decrease of the transport rate. These results confirmed the main conclusions of restricted diffusion theory, but did not supply the absolute values for the diffusion coefficient due to uncertainty in capillary geometry in synthetic membranes. The decreased mobility of ATP in the VDAC pore is in good agreement with the prediction of restricted diffusion theory. The hydrodynamic radius of ATP molecule is about 0.8 nm, the channel radius is about 1.3 nm. Their ratio is close to 0.6.

Theoretical models of restricted diffusion take into account properties of the bulk water. Do properties of water in a narrow pore differ from those in the bulk to the extent that they significantly affect diffusion? Bean and coauthors studied diffusion and pressure-driven flow of pure water through uniform and well defined pores with diameters in the range of 30–300 nm (Bean, 1972). They showed that their results are consistent with hydrodynamics of 'normal' (bulk) water and concluded that any rigid water layer must be only 1 nm or less in thickness. This conclusion is in a good agreement with observations by Gutman, Nachliel & Kiryati (1992) who studied proton diffusion in the interbilayer space and found no evidence for abnormal dynamics of protons. They have found that the diffusion coefficient of the proton in a 1–3 nm layer of water is nearly equal to its diffusion coefficient in bulk water. The latest molecular dynamics simulations performed at a total simulation time of 10^{-8} sec (Smith & Sansom, 1999) also point in this direction.

The effective short-time local diffusion coefficient of potassium and chloride ions was studied in a series of model ion channels. It was shown that mobility of both ions is appreciably reduced only in the narrowest channels. Specifically, a factor of 2 or greater mobility reduction was observed only for pore radii of 0.4 nm or smaller.

Large Molecules and Strong Interactions: Time-Resolved Single-Molecular Events

As discussed above, attractive and, probably, steric interactions between a particle and a pore are able to slow down particle passage through the pore dramatically. If these interactions are strong enough and/or translocating molecules are large, the event of a single-molecule passage can be resolved in time. One of the examples is the field-driven translocation of single-stranded RNA and DNA fragments through the α-toxin channel (Kasianowicz et al., 1996). Here, except for using voltage instead of pressure, the analogy to a Coulter counter is obvious.

This study employed synthetic polynucleotides of different molecular weights, which blocked current through the channel with the lifetimes proportional to their length. In particular, it was shown that at 120 mV applied to the membrane a 210-base poly[U] blocks the channel current for about 1 msec. A 200-base single-stranded polynucleotide has a length of about 100 nm and a diameter of about 1 nm, which means a diffusion coefficient of about 3×10^{-11} m^2/sec in the bulk water under normal conditions (Berg, 1993). Using this coefficient for the diffusion in the pore and applying the diffusion relaxation arguments presented above with the pore length substituted by the nucleotide length (the molecule is an order of magnitude longer than the channel length), we obtain 30 μsec for a characteristic time of a 'purely diffusional' relaxation. This estimate, that ignores the effects of the applied electric field altogether, is about 30 times smaller than the blocking time found experimentally (Kasianowicz et al., 1996). It is clear, however, that the electric field of about 10^7 V/m only decreases the passage time. Actually, it is the major driving force for nucleotide translocation. This conclusion follows from proportionality of the blocking time to the inverse of the applied potential and to the polymer length (and not to polymer length squared, as it would be in the case of diffusion relaxation). These findings, considered together with the above estimate, suggest strong polynucleotide/pore interactions.

Another example of time-resolved transport events is that of sugar transport through the solute-specific channel in the outer membrane of gram-negative bacteria, maltoporin. This channel, alternatively called LamB (Luckey & Nikaido, 1980), is known to be a part of the maltose uptake system (Szmelcman & Hofnung, 1975; Bloch & Desaymard, 1985; Nikaido, 1993). Maltoporin can be reconstituted in planar bilayer lipid membranes where it forms ion channels whose conductance is very sensitive to the presence of sugars in the membrane-bathing solution (Benz et al., 1986). These findings indicate that maltoporin contains the sugar-specific binding site that is located in the channel pore (Benz, Schmid & Vos-Scheperkeuter, 1987; Jordy, 1996; Andersen et al., 1999).

The kinetics of sugar binding to a site within maltoporin pore were studied with the help of noise analysis performed on multichannel membranes (Nekolla, Anderson & Benz, 1994; Andersen, Jordy & Benz, 1995). It was found that addition of sugars to the membrane-bathing solution decreased the membrane current in dose-dependent manner and also increased the spectral density of membrane current noise. The power spectral density of this sugar-induced noise could be approximated by a Lorentzian with the cutoff frequency that depended on sugar type and sugar concentration. This allowed calculation of the association and dissociation rates for the sugar binding reaction. The single-channel conductance derived from noise measurements was significantly smaller than the value measured in single-channel experiments (Nekolla et al., 1994). The authors speculated that the reason for such a discrepancy was related to the trimeric architecture of the maltoporin channel (Pavla & Westermann, 1979; Schirmer et al., 1995; Jap & Walian, 1998) and that one sugar molecule blocks a current only through one of the trimers.

Figure 6 shows time-resolved single-molecular events of maltohexaose binding to a maltoporin channel reconstituted into a planar lipid bilayer membrane. It is seen that at small sugar concentration (10 μM), current through the channel is randomly blocked with a characteristic time of several milliseconds to one third of the initial channel conductance. The probability of blocking is about 0.2. When sugar concentration is increased to 30 μM, states blocked to two thirds of initial conductance are clearly seen. A complete block of the channel can also be observed occasionally. The interpretation of such behavior is obvious. One sugar molecule blocks only one channel in the maltoporin trimer. When the sugar concentration is increased, the probability of simultaneous blocking grows to a value where double and triple blocking is made possible. Interestingly, one maltohexaose molecule is enough to block current through a single pore of the trimer completely.

Thus, because of a strong attractive interaction between the sugar molecule, maltohexaose, and the maltoporin pore, the effective mobility of this molecule inside the pore is slowed down by some five orders of magnitude in comparison to its bulk mobility. This circumstance permits observation of time-resolved single-

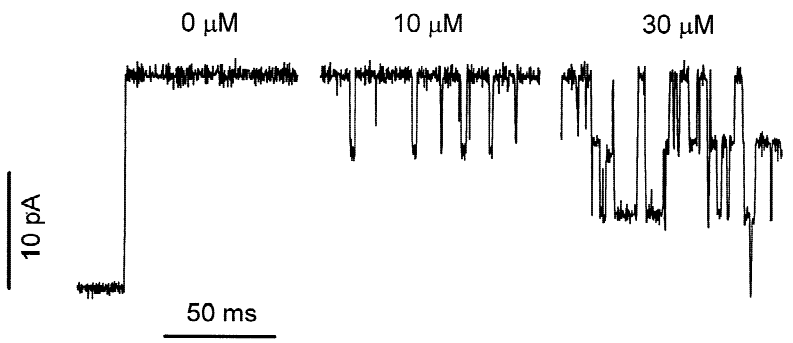

Fig. 6. Time-resolved single-molecular events of sugar exchange between the membrane-bathing solution and the maltoporin channel. Recordings of the current through a single maltoporin channel in the presence of 10 μM maltohexaose show current interruptions corresponding to one sugar molecule entry to one pore of the channel trimer. At 30 μM maltohexaose, two or even all three pores can be occupied by sugar simultaneously. Strong binding of maltohexaose to a site within maltoporin aqueous pore makes it possible to observe single-molecular events. Maltoporin was reconstituted into a planar lipid bilayer of 45 pF capacitance bathed by 1 M KCl at room temperature and pH 7.4. Currents were measured at 100 mV applied potential, time resolution was 0.2 msec. From S.M. Bezrukov and M. Winterhalter (*manuscript in preparation*).

molecular events. Other examples include protein-translocating channels of endoplasmic reticulum (Simon & Blobel, 1991) and nuclear pore complex channels in the presence of transcription factors or TATA-binding protein (Bustamante et al., 1995*a–c*). Figure 6 demonstrates the instructiveness of time-resolved observation of single-molecular events; however, if the molecular mobility is anywhere close to its value in the bulk, the corresponding time scale is too fast. For the ATP molecule average residence time in the VDAC pore we have about 10^{-7} sec (Rostovtseva & Bezrukov, 1998), which is far beyond modern resolution of single-channel experiments.

Conclusions

New methods for studies of channel-facilitated large-molecule transport are in great demand. It is well established that ion channels are key structural elements in metabolite exchange between different cellular compartments and between cells. Now, the evidence mounts that transmembrane channels also play a central role in protein trafficking (Simon & Blobel, 1991; Blobel, 1995; Simon, 1995). Moreover, a recent study (Marciano, Russel & Simon, 1999) suggests that particles as large as phage f1 (about 7 nm in diameter) can exit *Escherichia coli* host cells with the help of a phage-encoded channel protein. To understand the main principles and structural aspects of the large-molecule permeability and selectivity will require detailed information on transport phenomenology.

Molecular Coulter counters allow measurement of the pore-bulk exchange of higher molecule weight molecules such as metabolites and macromolecules, in the presence of small permeant ions. The method can be applied to a wide range of physiologically important solutes and a variety of different channels. It permits reliable quantitative evaluation of solute partitioning into, and dynamics within, the confines of aqueous pores of ion channels. However, as with any approach that does not trace the solute flux directly, it is model-dependent. Therefore, the method is most instructive when it is used in combination with other approaches which allow direct observation of transmembrane flux.

The molecular Coulter counter concept was largely inspired by the work of Charles Bean and his colleagues with submicron pores in polycarbonate films to count viruses and bacteriophage particles suspended in electrolyte solution (DeBlois & Bean, 1970; DeBlois et al., 1977). It also relies on the wealth of ion channel noise research (Conti & Wanke, 1975; Neher & Stevens, 1977; DeFelice, 1981; Fishman & Leuchtag, 1990), especially on studies of permeation of the slower moving ions by their blocking effect on the faster moving ions in the channel (Hess & Tsien, 1984; Heinemann & Sigworth, 1989). It is very close to the noise analysis method that is being developed for studies of sugar binding in porins (Nekolla, 1994; Andersen et al., 1995, 1998). The Coulter counter approach, however, demonstrates that one does not need any molecular binding to be able to access the main features of transport such as solute partitioning and dynamics in the channel pore. The effect on the channel conductance does not even have to be complete channel blocking. In the absence of strong binding, the diffusional considerations discussed above work well enough to describe the solute interference with small ion currents that reveals solute dynamics.

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